notes on methodology

Direct transesterification of all classes of lipids in a one-step reaction

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Summary Conventional techniques for the determination of fatty acid composition of lipids require solvent extraction, purification, hydrolysis, and derivatization procedures that are both lengthy and cumbersome. A 1-hr direct transesterification procedure carried out in methanol–benzene 4:1 with acetyl chloride circumvented all these steps and was applicable for analysis of both simple (triglycerides) and complex lipids (cholesteryl esters, phospholipids, and sphingomyelin). Recoveries (> 95%) of standards unaffected by the presence of 5% water and 200 mg of silica suggested that the technique could be used for the quantitative analysis of total fatty acids as well as of fatty acids in classes of lipids separated on silica from biological samples. When compared to the Folch procedure, the technique led to a 20.1% increase in total fatty acids for plasma, 3.9% for feces, 7.4% for bile, and 9.7% for rat liver. We therefore conclude that this one-step direct transesterification procedure is superior to currently used methods, not only because of its simplicity and speed, but also because of its added precision. —**Lepage, G., and C. C. Roy.** Direct transesterification of all classes of lipids in a one-step reaction. *J. Lipid Res.* 1986. **27:** 114–120.

Supplementary key words fatty acid • triglyceride • cholesteryl ester • phospholipid • sphingomyelin • gas-liquid chromatography • methylation

Fatty acid (FA) analysis of biological specimens by gas-liquid chromatography (GLC) requires solvent extraction, purification, and derivatization procedures that are both lengthy and cumbersome (1–3). Attempts to bypass extraction (4, 5) and purification steps (3, 6, 7) have met with varying degrees of success. This report proposes a technique that circumvents most of the preparative steps and consists of a one-step reaction. It leads to more complete recoveries of all classes of lipids which, during the transesterification procedure, are freed from biological specimens.

MATERIALS AND METHODS

Analytical grade solvents were redistilled in an all-glass system. All glassware was rinsed with chloroform–methanol 2:1 (v/v) and dried under nitrogen. Acetyl chloride (Fisher Scientific Ltd., Montreal, Quebec) was used without further purification. Borosilicate glass tubes with Teflon-lined screw-caps (100 × 13 mm) and magnetic stirring bars (10 × 3 mm) were bought from Fisher Scientific Ltd., Montreal, Quebec. Transesterification was carried out in a Reacti-Therm heating/stirring dry block which controls temperature ± 0.5°C (Pierce Chemical Co., Rockford, IL).

Fatty acid and fatty acid methyl ester standards (Analabs, North Haven, CT; Terochem, Rexdale, Ontario; Sigma, St. Louis, MO; and Mandel, Montreal, Quebec) as well as cholesteryl ester (CE), phosphatidylcholine, and chicken egg sphingomyelin (SP) standards (Sigma) were certified to be > 99% pure. Unsaturated lipid standards were bought packaged in ampoules under an inert gas to prevent oxidation.

Biological specimens that were analyzed (Table 4) consisted of plasma from a normal adult, fecal homogenate from a human milk-fed premature infant, bile from an adolescent with cerebrotendinous xanthomatosis, and rat liver homogenate.

Recoveries of standards as a function of time, the presence of silica, and added water

CE, PL, and SP standards were weighed and diluted with methanol–benzene 4:1 (v/v). After being mixed thoroughly, the mixtures of three standards were placed into individual reaction vials. These vials were submitted to the direct transesterification procedure described below for 1 hr, 4 hr, or 16 hr. To test whether silica could interfere with the reaction, 200 mg of silica was added to the mixture of standards. The liquid phase of this mixture was brought to a total volume of 2 ml with methanol–benzene 4:1 (v/v) and transesterification was carried out over periods of 1 hr to 16 hr. In order to check whether biological samples could be processed without prior extraction, the amount of water that could be added to standards without interfering with the direct transesterification procedure was tested in the following manner. One hundred μl, 200 μl, or 300 μl of water was added to standards, the solution was then mixed for 1 min, and the volumes were adjusted to 2 ml with methanol–benzene 4:1 (v/v) for the transesterification reaction. The samples

Abbreviations: GLC, gas-liquid chromatography; TG, triglyceride; CE, cholesteryl ester; PL, phospholipid; SP, sphingomyelin; FA, fatty acid.

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prepared in this manner contained 5%, 10%, or 15% of water, respectively. From the molecular weights of CE and PL standards and of their constituent FA, the theoretical 100% yield of fatty acid was calculated for each lipid standard. However, for the sphingomyelin solution, results obtained after 16 hr of transesterification with and without 5% water were taken as 100%.

Direct transesterification method

One hundred μl of either plasma or bile or 100 mg of either fecal or liver homogenate was precisely weighed in glass tubes. As shown in **Fig. 1**, an internal standard consisting of 50 μg to 300 μg of tridecanoic acid (C13:0), dissolved in 2 ml of methanol–benzene 4:1 (v/v) was precisely weighed and added to the biological samples. A small magnetic stirring bar was added to each tube and, while stirring, 200 μl of acetyl chloride was slowly added over a period of 1 min. Tubes were tightly closed with Teflon-lined caps and subjected to methanolysis at 100°C for 1 hr (8). Tubes were weighed before and after heating as a check for leakage. After tubes had been cooled in water, 5 ml of 6% K_2CO_3 solution was slowly added to stop the reaction and neutralize the mixture. The tubes were then shaken and centrifuged, and an aliquot of the benzene upper phase was injected into the chromatograph.

Extraction of biological samples by the Folch method (9, 10)

Biological specimens were processed as described earlier (11). After transesterification the pooled solvent extracts were dried under a gentle stream of nitrogen at room temperature. Residues were dissolved in 400 μl of hexane containing 50–300 μg of methylated tridecanoic acid used as an external standard. An aliquot was injected into the chromatograph.

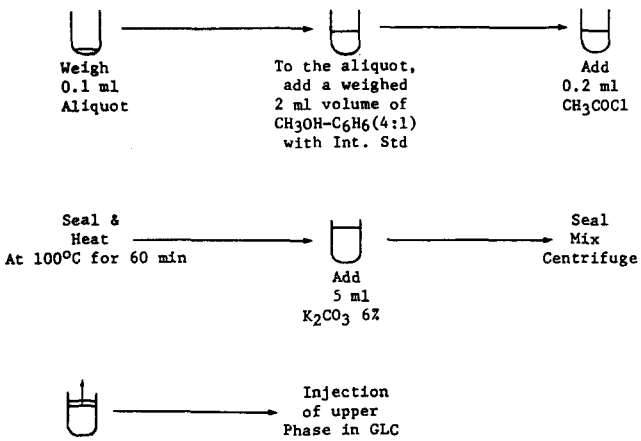

Fig. 1. Schematic diagram of the procedure for a biological sample.

Gas–liquid chromatography

FA were chromatographed as methyl esters on a 30-m fused silica column with an internal diameter of 0.32 mm (**Fig. 2**). The column was wall-coated with 0.20 mm SP-2330. Analysis was performed on a Hewlett-Packard 5880 gas chromatograph equipped with a flame ionization detector. Helium was used as carrier gas and nitrogen as make-up gas. The split ratio was 17:1. The injection port temperature was 200°C and the detector was 250°C. The column temperature was held at 80°C for 5 min and in a step-wise fashion reached a plateau of 220°C. The gas chromatograph was calibrated using a standard mixture of FA. A correction factor was applied to compensate for the lower ionization detector response to unsaturated FA relative to corresponding weights of saturated FA.

Calculations and validation of the method

In order to verify the purity and validate the exact concentration of both internal and external standards, they were alternately added to a known quantity of pentadecanoic acid (C15:0) which invariably yielded the same concentration on the chromatograph. Tridecanoic acid proved to be a reliable standard for biological samples since it invariably gave rise to a well-identified peak. It did not interfere with the FA patterns of plasma, bile, feces, and liver since they were shown to be free of endogenous tridecanoic acid.

RESULTS

Table 1 shows that a 1-hr period of transesterification at 100°C led to excellent recoveries of CE standards. Silica added to the standards neither interfered with the hydrolysis step nor with the esterification of the nascent FA. Addition of 100 μl of water to the standards had no effect on the completeness of the hydrolysis and methylation reactions achieved by the transesterification procedure carried out over a 1-hr period. However, when the solutions of CE standards were reconstituted with 10% or 15% of water there was impairment of the reactions. CE transesterification led to the appearance of three very polar peaks at the end of the chromatograms. Their surface area was directly related to the degree of hydrolysis that occurred in the absence or presence of water. After injection of methylated cholesteryl formate standard, it was concluded that these three peaks originated from the cleavage of cholesterol formate and FA. Attention should be drawn to the fact that one peak coeluted with the methyl ester of cerotic acid (C26:0) on the SP-2330 fused silica column.

Results with PL were similar to those obtained with CE in that transesterification was complete after 1 hr, and

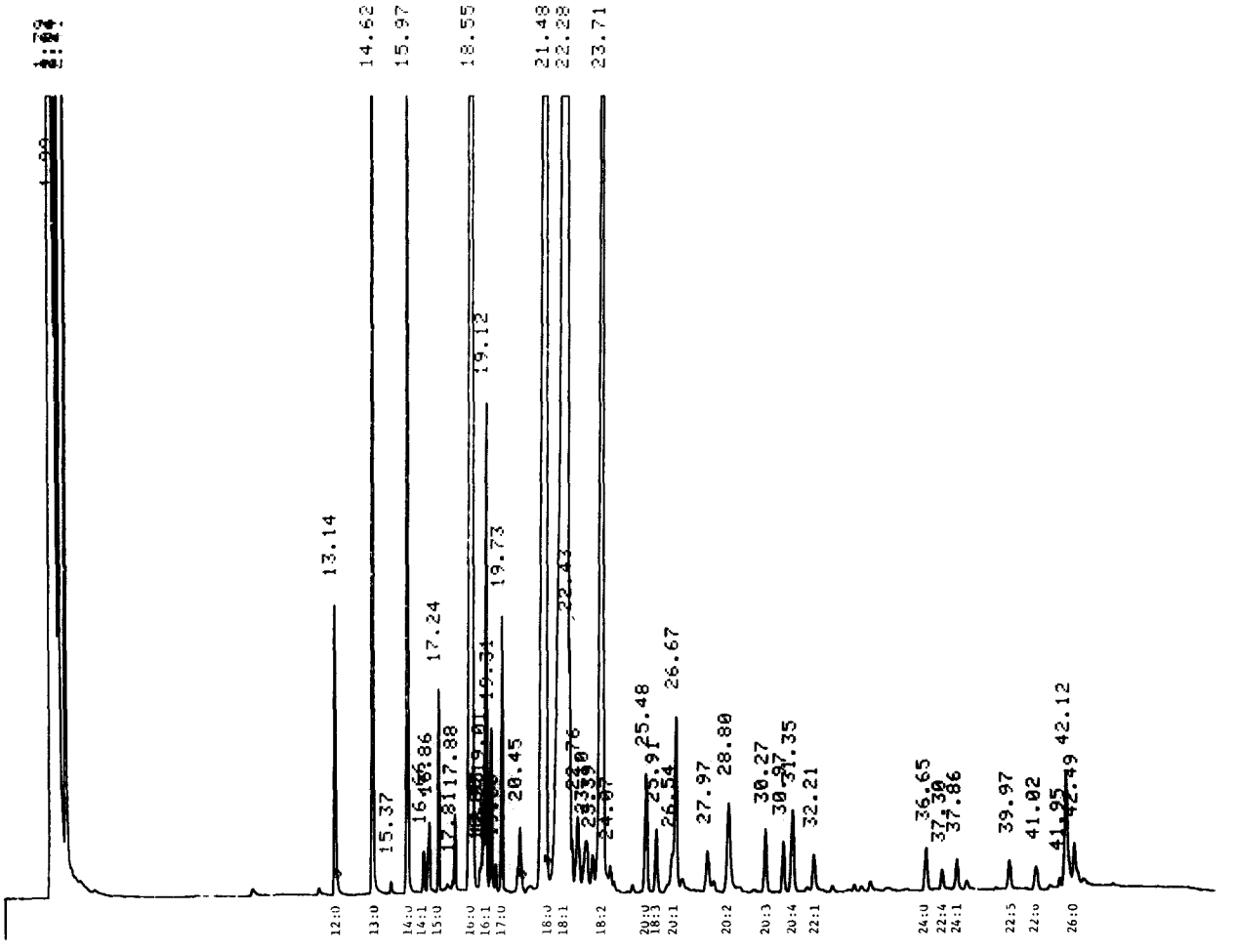

Fig. 2. Gas-liquid chromatography profile of fatty acid methyl esters (650 pg–250 ng of each) obtained from the fecal homogenate of a human milk-fed preterm neonate.

silica did not seem to have an adverse effect on the reaction (**Table 2**). However, in contrast to CE, neither 10% nor 15% water added to PL standards affected the percentage recovery of nascent methyl ester FA obtained after 1 hr.

In contrast to the results with solutions of CE and PL standards, hydrolysis of the SP solution was time-dependent in the sense that a transesterification reaction over a 4-hr period was advantageous (**Table 3**). Furthermore, the presence of silica affected the rate of the reaction to a significant extent. However, addition of 100 μl of water (5%) to the SP solution transesterified over a 1-hr period, even in the presence of silica, led to excellent recoveries of the nascent methyl ester FA. When the percentage of water was increased to 10% and 15%, less satisfactory results were obtained.

Table 4 compares individual fatty acids recovered from biological samples processed with the classical procedure

TABLE 1. The effect of time, silica and added water on direct transesterification of cholesteryl ester standards[a]

	No Added H_2O			Added H_2O[b]		
Fatty Acid	1 hr	4 hr	1 hr with Silica	5%	10%	15%
8:0	100.3	100.2	99.7	98.0	89.6	63.5
12:0	99.6	99.8	99.6	97.4	70.8	34.9
14:0	100.1	99.8	99.9	97.2	64.5	27.3
16:0	100.3	100.4	100.1	97.6	59.7	24.7
16:1 (n-7)	99.4	99.3	99.2	95.9	63.7	24.2
17:0	99.6	99.6	99.5	96.7	53.8	18.5
18:0	99.5	99.9	99.6	97.1	50.0	15.5
18:1 (n-9)	98.8	99.2	98.8	95.6	54.1	19.4
18:2 (n-6)	99.2	99.6	99.2	95.7	58.2	22.5
18:3 (n-3)	98.0	98.5	98.3	94.6	61.3	25.5
20:4 (n-6)	95.7	96.9	95.2	92.4	53.4	20.9

[a] Values are expressed as % recovery and represent means of two samples.
[b] One hr transesterification carried out with added H_2O.

TABLE 2. The effect of time, silica and added water on direct transesterification of phospholipid standards[a]

	No Added H_2O			Added H_2O[b]		
Fatty Acid	1 hr	4 hr	1 hr with Silica	5%	10%	15%
12:0	100.6	100.1	100.1	100.6	99.5	98.4
14:0	99.1	99.4	97.9	98.3	97.7	96.0
16:0	98.7	99.8	96.8	97.8	96.5	95.5
17:0	98.5	99.8	96.6	97.7	96.1	95.3
18:0	98.0	99.6	96.2	97.2	95.4	94.9
18:1 (n-9)	98.4	99.8	96.0	97.6	96.5	95.1
18:2 (n-6)	99.1	100.5	96.7	98.2	96.7	96.0
20:0	96.4	98.1	94.5	95.9	94.2	93.2

[a]Values are expressed as % recovery and represent means of two samples.
[b]One hr transesterification carried out with added H_2O.

of Folch, Lees, and Sloane Stanley (9) and the direct transesterification method. Aliquots of plasma, feces, bile, and liver were added with internal standard dissolved in methanol–benzene 4:1 (v/v) to achieve a final water concentration of less than 5%. The comparative FA content was higher with the direct transesterification technique for all specimens. The percentage increase of total FA recovered was 20.1% for plasma, 3.9% for feces, 7.4% for bile, and 9.7% for liver.

The stability of the methyl ester FA in the benzene upper phase was excellent. A weekly injection of the same sample stored at 4°C gave identical results after 3 months. Losses of unsaturated FA by oxidative processes, during the direct transesterification reaction or following the 3-month period of storage were never observed.

DISCUSSION

As we pointed out recently for triglycerides (11), this one-step reaction is rapid and reproducible. During the initial phase of our study we tried to transesterify CE, PL, and SP standards with 100 μl of acetyl chloride using the same technique as with FA and TG standards. However, poor results were obtained especially for SP which underwent only ± 55% hydrolysis. This was not surprising in view of the observations of MacGee and Williams (12) that sphingolipids are extremely resistant to alkaline treatment. Sphingolipid fatty acids exist as amides rather than esters, and sphingolipids undergo acid hydrolysis or acid methanolysis very slowly. After testing up to 350 μl of acetyl chloride, 200 μl was found to be sufficient to hydrolyze and methylate all lipid classes. To our surprise it was noted that transesterification of SP was helped by the presence of 5% water. This may very well be due to the fact that SP are amphipathic (13). From our experience with the measurement of FA content of SP in both standards and biological specimens, it may be concluded that the technique described is reliable without extraction and requires only 1 hr as opposed to 1.5 to 2 hr for the hydrolytic phase alone, once extraction of SP from tissues has been carried out (12, 14).

Because recoveries of linoleic, homo-gamma linolenic, and arachidonic acids are temperature-dependent, we tried working at a lower temperature as recommended by Haan, Van Der Heide, and Wolthers (3). This failed as only 65% hydrolysis took place when the reaction was carried out at 70°C for 2 hr. Moreover, the ratio of polyunsaturated/saturated FA did not improve over that obtained at 100°C. It was then concluded that loss of the polyunsaturated FA at high temperatures is associated with saponification procedures rather than acid hydrolysis.

Different proportions of methanol–benzene were also studied. It appeared that larger amounts of benzene did not help the transesterification. In fact, decreasing the percentage of benzene from 20% to 10% led to recoveries of only 20% for palmitoleic and arachidonic, 26% for linoleic, and 30% for oleic. No change was observed in the recoveries of saturated FA. The use of 400 μl of benzene present in the 2 ml of benzene–methanol (4:1) supplied a sufficiently concentrated upper phase extractant after transesterification to allow direct injection into the chromatographic unit, circumventing thereby the need for a time-consuming and ineffiicient evaporation step.

In view of the consistent recoveries (96–100%) of the three internal standards used in the report recently published (11), we elected to use only one standard, namely tridecanoic acid, which efficiently monitors the completeness of the extraction of methyl ester FA into the benzene phase after transesterification. Pentadecanoic (15) and heptadecanoic acids (16) have also been used but tridecanoic acid was selected because it was the only one totally absent from biological samples studied. A further advantage is that it forms a well-individualized peak in a relatively uncrowded part of the fatty acid methyl ester chromatographic run.

The addition of 5 ml of potassium bicarbonate should be done slowly because of the quick liberation of CO_2

TABLE 3. The effect of time, silica and added water on direct transesterification of sphingomyelin standard[a]

	No Added H_2O			Added H_2O[b]			
Fatty Acid	1 hr	4 hr	1 hr with Silica	5%	10%	15%	5% with Silica
14:0	90.5	94.6	87.8	97.3	89.2	91.9	98.6
16:0	92.7	97.8	85.4	97.9	91.2	92.7	98.1
18:0	94.1	98.4	87.5	99.2	88.8	94.6	95.7
18:1 (n-9)	92.9	100.0	92.9	103.6	89.3	85.7	100.0

[a]Values are expressed as % recovery and represent means of two samples.
[b]One hr transesterification in the presence of water with or without silica.

TABLE 4. Comparative fatty acid content of biological specimens using the classical technique of Folch et al. and the direct transesterification method[a]

Fatty Acid	Plasma		Feces		Bile		Liver	
	Folch et al. Extraction	Direct Transesterification[b]	Folch et al. Extraction	Direct Transesterification	Folch et al. Extraction	Direct Transesterification	Folch et al. Extraction	Direct Transesterification
	μg/ml		*mg/72 hr*		*μg/ml*		*μg/g*	
12:0			32 ± 0.3	40 ± 0.3				
14:0	20 ± 0.6	25 ± 0.3	148 ± 1.1	160 ± 0.5	59 ± 1.8	66 ± 0.5	trace	15 ± 0.1
14:1 (n-5)			4 ± 0.1	4 ± 0.1				
15:0			19 ± 0.2	20 ± 0.1	44 ± 0.9	48 ± 0.2		
16:0	444 ± 1.7	522 ± 2.5	993 ± 7.7	1035 ± 7.5	3064 ± 10.5	3259 ± 18.6	1522 ± 11.5	1668 ± 10.2
16:1 (n-7)	25 ± 0.1	31 ± 0.5	53 ± 0.4	56 ± 0.4	226 ± 1.7	243 ± 2.2	38 ± 0.3	41 ± 0.3
17:0			28 ± 0.3	29 ± 0.2	26 ± 0.2	28 ± 0.1	45 ± 0.5	49 ± 0.4
18:0	158 ± 0.6	186 ± 0.1	709 ± 7.2	733 ± 5.8	317 ± 1.0	338 ± 2.4	2059 ± 18.1	2216 ± 15.3
18:1 (n-9)	374 ± 11.5	444 ± 11.9	1390 ± 15.0	1432 ± 10.4	1087 ± 2.7	1153 ± 9.8	541 ± 7.5	576 ± 4.6
18:2 (n-6)	831 ± 4.1	968 ± 3.8	307 ± 4.4	313 ± 2.8	1897 ± 6.2	2046 ± 15.4	1601 ± 14.7	1718 ± 12.5
18:3 (n-3)			14 ± 0.2	15 ± 0.1	40 ± 0.2	43 ± 0.3		
20:0			14 ± 0.2	15 ± 0.1				
20:1 (n-9)			23 ± 0.5	24 ± 0.2				
20:2 (n-6)			20 ± 0.5	20 ± 0.1				
20:3 (n-6)	33 ± 0.4	41 ± 0.5	13 ± 0.2	13 ± 0.1	198 ± 0.9	217 ± 1.9	102 ± 1.8	112 ± 0.8
20:4 (n-6)	165 ± 0.8	201 ± 1.1	17 ± 0.2	19 ± 0.2	720 ± 3.5	788 ± 6.1	1878 ± 19.3	2042 ± 15.4
22:0							trace	22 ± 0.4
22:1 (n-9)			7 ± 0.1	8 ± 0.1				
22:4 (n-6)			4 ± 0.1	4 ± 0.1			21 ± 0.2	23 ± 0.2
22:5 (n-6)			6 ± 0.1	6 ± 0.1			95 ± 1.0	105 ± 1.0
22:6 (n-3)			5 ± 0.1	6 ± 0.2	104 ± 0.4	118 ± 0.9	883 ± 9.8	970 ± 7.9
24:0	trace	20 ± 0.2	8 ± 0.1	10 ± 0.1			42 ± 1.3	76 ± 0.5
24:1 (n-9)	trace	25 ± 0.4	6 ± 0.2	6 ± 0.1			15 ± 0.1	27 ± 0.5
26:0			12 ± 1.2	15 ± 0.5	74 ± 3.3	89 ± 4.3	56 ± 3.3	77 ± 2.8
Total	2051 ± 15.0	2463 ± 20.1	3810 ± 37.9	3957 ± 28.4	7856 ± 21.6	8436 ± 55.4	8871 ± 97.0	9730 ± 64.9

[a] Mean ± SE of five samples from the same specimens.
[b] See text for details.

bubbling the mixture. Addition of this strong base is necessary to bring the pH back to neutral. This is an important step in the procedure because, after transesterification with 200 μl of acetyl chloride, the pH of the solution is less than 1 and injection of this acidic benzene supernatant can break down the very thin stationary phase coating the capillary column which is thereby rendered useless. Rogiers (17) and Christie (18) have reported the same problem.

The experiments carried out with silica were important in validating the usefulness of the method for the analysis of various lipid classes after their separation on silica. Following the thin-layer chromatographic separation of lipid classes on silica of Bitman, Wood, and Ruth (19), bands can be scraped from the plate and added directly to the 2 ml of transesterification mixture with excellent recoveries. In fact, we found that up to 200 mg of silica can be used without concern that it will in any way interfere with the direct transesterification method.

In an attempt to improve recoveries of methyl ester FA migrating in the benzene upper phase after transesterification, solvents such as pentane, hexane, or isooctane were added but were not beneficial. Chromatographic profiles on 30 M SP-2330 capillary columns were remarkably good. Since the glassware was thoroughly cleaned and redistilled solvents were used, no artefact peaks were seen on blank runs and solvent peaks were narrow without trailing. Furthermore, baseline resolution was obtained with all fatty acids including the polyunsaturated fatty acids which on some columns are reported to coelute (20). After processing hundreds of standard mixtures and specimens and injecting them into the gas chromatograph, no alteration of the chromatographic column has so far been observed.

The comparative FA content of biological specimens was higher with the direct transesterification technique than that obtained using the classical technique of Folch et al. (9). The percentage increases of total FA in plasma, feces, bile, and liver were 20.1%, 3.9%, 7.4%, and 9.7%, respectively. The physicochemical properties of classes of lipids and their varying distributions in biological specimens tested most likely explain the fact that the degree of discrepancy between the two methods is not the same for all samples.

The Folch extraction takes advantage of the low solubility of lipids and of their preference for water-immiscible organic solvents, conferred by the long side chains of FA. Since membrane and plasma lipids are normally associated with proteins, Folch et al. (9) used chloroform–methanol 2:1 (v/v). Because of its water solubility and

hydrogen-bonding ability it induces splitting of the lipid–protein complex and even in some cases denatures the protein.

There was little advantage (+ 3.9%) in the direct transesterification method over the Folch extraction in the FA recovery from feces. This is likely due to the fact that 84.5% of the lipids were in the form of free fatty acids and the rest was almost entirely made up of TG that are easily extracted from a water solution with a nonpolar solvent such as the one used by Folch et al. (9). Fasting plasma lipids are mostly made up of CE and PL. As water is strongly hydrogen bonded to protein and to polar lipids in lipoproteins, covalently bound lipids must be subjected to a hydrolysis procedure before they can be extracted completely with organic solvents of any polarity (21). We feel that these bound lipids likely account for the higher discrepancy (+ 20.1%) found between the two methods for plasma. In terms of physical properties, PL "bridge the gap" between the completely water-insoluble neutral lipids and molecules that form true aqueous solutions. The amphipathic character of PL makes them more easily extractable than CE (13). PL are the major lipid constituents of bile and liver (22). Bolton et al. (23) encountered serious problems in the lipid analysis of bile after Folch extraction. They proposed that biliary PL were subject to some error because water-soluble PL such as lysolecithin were removed by aqueous washes (23). A 7.4% discrepancy for bile between the two methods therefore seems reasonable. Lipids bound to membranes should be taken into account for the higher (9.7%) difference found for liver homogenate. From the above discussion, it may be concluded that the more complete recovery of FA from complex lipids is due to the fact that they were directly and completely freed from biological specimens during the transesterification procedure.

The determination of FA composition is conventionally done by GLC. It requires solvent extraction, purification, hydrolysis, and derivatization procedures that are both lengthy and cumbersome (1–3). The proposed method provides a total FA profile after a one-step reaction without losses and in less time than that required for a gravimetric determination (24). Furthermore, the FA pattern of various lipid classes may be easily obtained following TLC and direct transesterification of the silica scrapings since silica does not interfere with the reaction.

Hundreds of biological specimens have now been run in duplicate by the direct transesterification method. The coefficient of variation for the different FA varied from 0.4 to 2.0% for several samples that were processed at least five times each. The proposed technique gave better results than the 3% to 11.8% coefficient of variation reported by MacGee and Williams (12). Although simple and rapid, it is also more precise than currently used techniques because it bypasses extraction and purification steps and also calls for the addition of an internal standard at the beginning. However, good reproducibility can only be expected if both the specimen to be analyzed and the internal standard are carefully weighed.

In conclusion, the direct transesterification technique described recently for FA and TG (11) has been modified and is now applicable to both simple and complex lipids. Because of its simplicity, speed, and added precision, it has proved to be very useful in our hands and should attract the attention of lipidologists.

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